application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Amendments

In the Title:

Please substitute the following Title of the Invention for the pending Title of the Invention:

DNA Sequence Encoding a Novel Glucuronyl C5-Epimerase.

In the Specification:

Please substitute the following paragraphs/sections for the pending paragraphs/sections.

Substitute the section heading beginning on page 3, line 15, with the following section heading:

–Brief Description of the Figures--

Substitute the paragraph on page 3, starting with line 7, with the following paragraph:

-- Specific DNA sequences according to the invention are defined in the Sequence Listing.--

D'

Substitute the two paragraphs on page 5, starting at line 32 with the following paragraphs:

-- *Peptide Purification and Sequencing* - The 52 kDa epimerase protein (~1 μg), purified from a detergent extract of bovine liver by chromatography on O-desulfated heparin-SEPHAROSE, RED-SEPHAROSE, Phenyl-SEPHAROSE, and Concanavalin A-SEPHAROSE (Campbell, P., Hannesseon, H.H., Sandbäck, D., Rodén, L., Lindahl, U and Li, J-p. (1994) J Biol Chem **269**, 26953-26958), was subjected to direct N-terminal sequencing using a model 470A protein sequenator (Applied Biosystems) equipped with an on-line 120 phenylthiohydantoin analyzer (Tempst, P., and Riviere, L. (1989) Anal. Biochem. **183**, 290-300). Another sample (~1μg) was applied to preparative (12%) SDS-PAGE and was then transferred to a PVDF membrane. After staining the membrane with Coomassie Blue, the enzyme band was excised. Half of the material was submitted to direct N-terminal sequence analysis, whereas the remainder was digested with Lys-C (0.0075 U; Waco) in the presence of 1% RTX-100/10% acetonitrile/100mM Tris-HCL, pH 8.0. The generated peptides were separated on a reverse phase C4-column, eluted at a flow rate of 100 μl/min with a 6-ml 10-70% acetonitrile gradient in 0.1% trifluoroacetic acid, and detected with a 990 Waters diode-array detector. Selected peptides were then subjected to sequence analysis as described above.-

D2

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Probes for Screening - Total RNA was extracted from bovine liver according to the procedures of Sambrook *et al.* (1989). Single-stranded cDNA was synthesized by incubating ~5 μg of bovine liver total RNA (denatured at 65°C, 3 min) with a reaction mixture containing 1 unit RNAse inhibitor (Perkin-Elmer Corp.), 1 mM of each dNTP, 5 μM random nucleotide hexamer and 1.25 units of murine leukemia virus reverse transcriptase (Perkin-Elmer Corp.) in a buffer of 10 mM Tris-HCL, pH 8.3. The mixture was kept at 42°C for 45 min and then at 95°C for 5 min. Degenerated oligonucleotide primers were designed based on the amino-acid

sequence determined for one of the internal peptides derived from the purified epimerase (Table 1). Single-stranded bovine liver cDNA was applied to PCR together with 100 pmols of primers 1 (sense) and 3 (antisense), in a total volume of 100 µl containing 1µl of 10% Tween 20, 6 mM $MgCl_2$, 1 mM of each dNTP, and 2.5 units TAQ polymerase (Pharmacia Biotech) in a buffer of 10 mM Tris-HCL, pH 9.0. The reaction products were separated on a 12% polyacrylamide gel. A ~100-bp band was cut out from the gel and reamplified using the same PCR conditions. After an additional polyacrylamide gel electrophoresis, the product was isolated and sequenced, yielding a 108-bp sequence. This PCR product was subcloned into a pUC119 plasmid. The DNA fragment cleaved from the plasmid was labeled with [^{32}P] dCTP (DuPont NEN) using a Random Primed DNA Labeling Kit (Boehringer Mannheim).--

D2 cont.

Substitute the four paragraphs on page 7, starting with line 25, and ending on page 9, line 8 with the following paragraphs:

-- *Subcloning and Sequencing of cDNA Inserts* - cDNA inserts, isolated by preparative agarose gel electrophoresis (Sambrook *et al.*, 1989) after *Eco*RI restriction cleavage of recombinant bacteriophage DNA, were subcloned into a pUC119 plasmid. The complete nucleotide sequence was determined independently on both strands using the dideoxy chain termination reaction either with [^{35}S]dATP and the modified T7 polymerase (SEQUENASE version 2.0 DNA sequencing Kit; U. S. Biochemical Corp.) or the ALF™ System (Pharmacia Biotech). DNA sequences were compiled and analyzed using the DNASTAR™ program (Lasergene).

D3

Polyclonal Antibodies and Immunodetection - A peptide corresponding to residues 77-97 of the deduced epimerase amino-acid sequence was chemically synthesized (Åke Engström,

Department of medial and Physiological Chemistry, Uppsala University, Sweden), and was then conjugated to ovalbumin using glutaraldehyde (Harlow, E. and Lane, D., (1989) in Antibodies: A laboratory Manual, pp 78-79, Cold Spring Harbor, NY). A rabbit was immunized with the peptide conjugates together with Freund's adjuvant. After 6 boosts (each with 240 μg conjugated peptide) blood was collected and the serum recovered. The antibody fraction was further purified on a Protein A-SEPHAROSE column (Pharmacia Biotech), and used for immunoblotting.

Samples of GlcA C5-epimerase were separated under denaturing conditions by 12% SDS-PAGE, and were then transferred fo a nitrocellulose membrane (HYBOND™ ECL). ECL immunoblotting was performed according to the protocol of the manufacturer (Amersham). Briefly, the membrane was first treated with blocking agent, then incubated with purified antibody, and finally incubated with the peroxidase labeled anti-rabbit antibody. After adding the ECL reagent, the light emitted by the chemical reaction was detected to HYPERFILM™ ECL for 30-60 sec.

D3 cont.

Northern Blot Hybridization - Bovine liver and lung total RNA was prepared according to Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY), and mouse mastocytoma (MCT) total RNA was extracted from a tumor cell line (Montgomery, R.I., Lidholt, K., Flay, N.W., Liang, J., Verter, B., Lindahl, U. and Esko, J.D. (1992) PNAS **89**, 11327-11331) as described by Chromczynski and Sacci (1987). Total RNA from each tissue (~20 μg samples) was denatured in 50% formamide (v/v), 5% formaldehyde, 20 mM Mops buffer, pH 7.0, at 65 ^{0}C for 5 min. The denatured RNA was separated by electrophoresis in 1.2% agarose gel containing 5% formaldehyde (v/v), and was then transferred to a HYBOND

N^+ nylon membrane (Amersham). The RNA blot was pre-hybridized in EXPRESSHYB Hybridization Solution (Clontech) at 65 ^{0}C for 1 h, and subsequently hybridized in the same solution with a [^{32}P]dCTP-labeled DNA probe (a 2460 bp fragment including the 5'-end of the cDNA clone; see the sequence listing). The membrane was washed in 2x SSC, 0.5% SDS at the same temperature for 2 x 15 min and in 0.5x SSC, 0.5% SDS for 2 x 5 min. The membrane was exposed to Kodak X-ray film at -70^{0}C for 24 h.--

D3 wrch.

Substitute the two paragraphs on page 12, starting with line 19, with the following paragraphs:

-- *Characterization of cDNA and predicted protein structure* - The total cDNA sequence identified, in all 3085 bp, contains an open reading frame corresponding to 444 amino-acid residues (SEQ ID NO: 13). Notably, the coding region (1332 bp) is heavily shifted toward the 5'-end of the available cDNA, and is flanked toward the 3'-end by a larger (1681 bp) noncoding segment. The deduced amino-acid sequence corresponds to a 49,905 dalton polypeptide. All of the five peptides isolated after endo-peptidase digestion (Table I) were recognized in the primary structure deduced from the cDNA (SEQ ID NO: 12). One of these peptides (peptide 1) is identical to the N-terminus of the isolated liver protein. This peptide was found to match residues 74-86 of the deduced polypeptide sequence (SEQ ID NO: 13). The enzyme isolated from bovine liver thus represents a truncated form of the native protein.

D4

Generation of mRNA from an expression vector inserted with the 3-kb cDNA clone, followed by incubation of the product with rabbit reticulocyte lysate in the presence of [^{35}S] methionine, resulted in the formation of a distinct labeled protein with an estimated M_r of ~50 kDa (Fig. 1). This product was recognized in immunoblotting (data not shown) by polyclonal

D4 corr.

antibodies raised against a synthetic peptide corresponding to residues 77-79 (SEQ ID NO: 13) of the deduced amino-acid sequence. The same antibodies also reacted with the isolated ~52 kDa bovine liver protein (data not shown). These observations establish that the 3-kb cDNA is derived from the transcript that encodes the isolated ~52 kDa bovine liver protein.--

Substitute the paragraph on page 13, starting with line 13, with the following paragraph:

D5

-- The cDNA structure indicates the occurrence of 3 potential N-glycosylation sites (Table III). Sugar substituents may be important for the proper folding and catalytic activity of the enzyme, since the protein expressed in bacteria (which also gave a strong Western signal towards the polyclonal antibodies raised against the synthetic peptide; data not shown) was devoid of enzymatic activity. A potential transmembrane region is underlined in Table III. The predicted protein contains two cystein residues, only one of which occurs in the isolated (truncated) protein. Since NEM was inhibitory to epimerase activity (data not shown), this single cystein unit may be essential to the catalytic mechanism.--

On page 18, line 1, please delete "SEQUENCE LISTING" and substitute –Table III-- therefor.

In the sequence Listing:

If not already done in response to the amendment and reply mailed 4/13/2001, please enter pages 1-9 of the sequence listing submitted on 4/13/2001.

In the Claims: